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Reversing Anoikis Resistance in Triple-Negative Breast Cancer

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14. ABSTRACT During the first year of this idea expansion grant, we have addressed Aim1 , which was to determine if restoration of miR-200c and inhibition of miR-222 can enhance TNBC differentiation in 3D culture. We found addition of miR-200c and miR-222 inhibition make TNBC colonies in culture in 3D in matrigel smaller, rounder and increase Dicer protein. Additionally, restoration of miR-200c decreases the amount of xCT protein, which regulates intracellular glutathione levels). There are multiple predicted target sites for both miR-200c and miR-193 in the xCT 3'UTR so we are cloning this region to prove that it is a direct target of these miRNA since miR-200c is lost in TNBC that are anoikis resistant and miR-193b decreases in suspension. We also find that restoration of miR-200c increases epithelial splicing factors in TNBC. In this first year we have also completed tasks in Aim 2 to identify the mechanisms by which TNBC cells resist anoikis. We confirmed that TNBC regulators of reactive oxygen species such as xCT and stress and inflammatory pathway genes including COX2 in suspension. Lastly, we followed up on our observation that components of the kynurenine pathway (KP), that leads to formation of NAD(+) increases in suspension. We demonstrate that knockdown of COX2 increases death in suspension as does knockdown or inhibition of TDO2. Our study continues to generate mechanistic and pre-clinical data necessary to determine if manipulation of key miRNAs has potential as a form of "differentiation therapy" for TNBC, for which there is currently no effective targeted treatment.					
15. SUBJECT TERMS Breast cancer, androgen receptor, estrogen receptor, growth factors, enzalutamide, endocrine resistance, targeted therapy					
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1. INTRODUCTION:

For this idea expansion grant, **we hypothesized that loss of miR-200c and overexpression of miR-222 are largely responsible for the dedifferentiated phenotype and aggressive behavior of claudin low TNBC and metaplastic breast cancers, and that manipulation of these miRNA or key targets could render such tumors less aggressive and more treatable.** Addressing Aim1, to determine if restoration of miR-200c and inhibition of miR-222 can enhance TNBC differentiation in 3D culture, we have found that addition of miR-200c and miR-222 inhibition make TNBC colonies in 3D culture in matrigel smaller, rounder and increase Dicer protein. Additionally, restoration of miR-200c decreases the amount of xCT protein, which regulates intracellular glutathione levels. There are multiple predicted target sites for both miR-200c and miR-193 in the xCT 3'UTR so we are cloning this region to prove that it is a direct target of these miRNA, since miR-200c is lost in TNBC that are anoikis resistant, and miR-193b decreases in suspension. We also find that restoration of miR-200c increases epithelial splicing factors in TNBC. In this first year we have also completed tasks in Aim 2 to identify the mechanisms by which TNBC cells resist anoikis. We confirmed at the protein level, that TNBC upregulate regulators of reactive oxygen species such as xCT and stress and inflammatory pathway genes including COX2 and downstream effector levels of prostaglandin E(2) (PGE(2)), also increase in suspension. Lastly, we have also followed up on our observation that components of the kynurenine pathway (KP), such as the rate limiting enzyme TDO2 (that leads to production of the essential pyridine nucleotide, nicotinamide adenine dinucleotide (NAD(+))) increase in suspension. A metabolite of this pathway, kynurenine, and the receptor that it binds to, the arylhydrocarbon receptor, also increase in suspension. These factors contribute to the ability of TNBC to resist anoikis and be more metastatic in vivo. We demonstrate that knockdown of COX2 increases death in suspension as does knockdown or inhibition of TDO2 or AhR.. Our study continues to generate mechanistic and pre-clinical data necessary to determine if manipulation of key miRNAs has potential as a form of “differentiation therapy” for TNBC, for which there is currently no effective targeted treatment. The work in this study may have great impact on the field of breast cancer and hopefully oncology practice eventually because we have identified genes and their protein products that TNBC make only when they are in suspension. These proteins may function to help TNBC survive in suspension while in transit in the blood or lymphatic system to cause metastases. Identification of these genes/proteins was not possible via mining of the currently available data bases such as TCGA, which are performed with RNA from the primary tumor, not tumor cells in circulation. Furthermore, we find that some of these genes/proteins are regulated by miRNA, such their change in suspension is more apparent at the protein level than at the genes level. Knowledge of these proteins that are facilitating survival in suspension, may provide unique, previously unidentified targets for TNBC therapy.

2. KEYWORDS: Breast cancer, anoikis resistance, xCT, COX2, TDO2, survival in suspension, metastasis, triple negative breast cancer (TNBC)

BODY:

3. ACCOMPLISHMENTS: Below we describe for each task in the official statement of work the major activities; specific objectives; significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or other achievements. We include discussion of stated goals not met or tasks not fully completed. We include pertinent data and graphs in sufficient detail to explain significant results achieved. We have three manuscripts drafts in preparation almost ready to submit that relate to this project.

The objective of Aim 1 of this proposal is to determine if restoration of miR-200c and inhibition of miR-222 in TNBC can enhance differentiation in 3D culture and render TNBC more like the less aggressive luminal subtype.

What was accomplished under these goals?

Task 1 Months 1-3. (50% completed). Our objective was to determine if miR-200c is able to restore apical-basal polarity to TNBC cell lines.

Results: Adding miR-200c mimic causes TNBC cells, which usually form “stellate” structures in 3D culture cells, to form rounded balls reminiscent of the luminal subtype of breast cancer in 3-D culture (Figure 1).

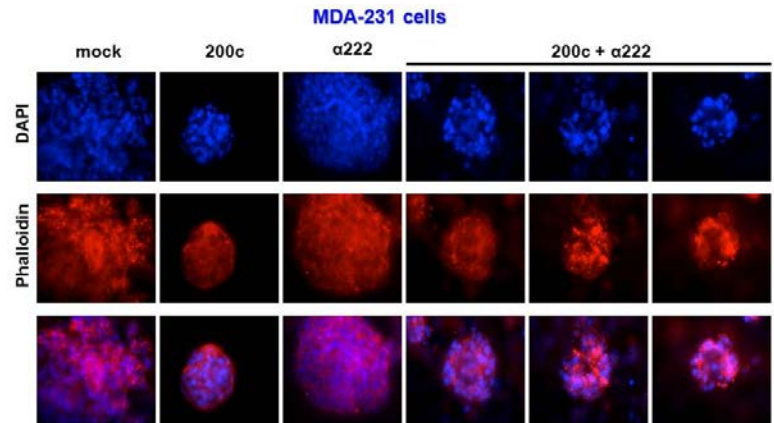


Figure 1. Effects of restoration of miR-200c and inhibition of miR-222 on MDA-231 cells in 3D culture.

MDA-231 cells were mock transfected or transfected with 50 μ M miR-200c mimic, 50 μ M miR-222 antagomiR or 50 μ M miR-200c mimic plus 50 μ M miR-222 antagomiR. Cells were then plated in growth factor reduced Matrigel and allowed to grow for 7 days at which time they were fixed and stained with phalloidin (to show actin localization and thus cell structure) and DAPI (to show nuclei).

MiR-200c restoration alone reduces the size of the stellate structures and renders them smaller while antagonizing miR-222 alone just makes the organoids more round. Both together appears to make the structures start to hollow out. We have not been able to finish this task because we need to out optimal conditions since restoration of miR-200c reduces proliferation, we have to optimize the length of time and how many cells to put into matrigel to get enough colonies and maximum differentiation. Then we can get enough colonies to stain for the polarity proteins and markers of apoptosis to see if the structures are differentiating to form hollow duct-like structures. This links into task 2 in which we tried this again and paraffin embedded the structures and stained for Dicer since we previously published that both restoration of miR-200c and antagonizing miR-222 increase Dicer levels.

Task 2. Months 4-5. (75% completed) Determine if the protein products of targets that we know to be relevant in TNBC and change when we re-introduce miR-200c alone or combine with inhibition of miR-222 activity in 3D culture We performed IHC for Dicer in these cells and do find it to be increased most when miR-200c is restored and the lentiviral antagomer for miR-222 is present (Figure 2). We generated BT549 cells stably transduced with lentiviral vector expressing miR-200c under the control of a tetracycline inducible promoter (p-TRIPZ) we can also put miR-222 antagomiR (pMiR-222-ZIP) or scrambled control into the cells. We induced with doxycyclin and infected with antagomiR (pMiR-222-ZIP lentivirus) before cells were plated on growth factor reduced Matrigel and then cultured as shown in Figure 2.

BT549 3D Culture
Dicer IHC 100X



Figure 2. Dicer increases in TNBC by the UCDenver Tissue Biobanking and Processing Core. Cut sections on slides were deparaffinized, antigens were heat retrieved in 10mM citrate buffer, pH 6.0, and endogenous peroxidase quenched in 3% H2O2. Rabbit anti-Dicer antibody 5818 (Abcam, Cambridge, MA) and the Vectastain Universal Elite ABC kit (Vector Laboratories, Burlingame, CA) was used for antibody detection.

Interestingly, we find that Dicer protein as detected by immunohistochemistry (IHC) is dramatically reduced in TNBC clinical specimens as compared to adjacent normal epithelium, while this is not the case in ER+ disease (Figure 3), and we have previously published that miR-200c is reduced in TNBC and miR-222 elevated in TNBC, but not in ER+ tumors (1). We have a draft of a paper regarding the detection of Dicer in breast cancer by IHC since there are a bunch of antibodies that give very poor results and we had to determine which one was best and develop the protocol. The information regarding this paper that is almost ready to send for review is provided later in this progress report.

A

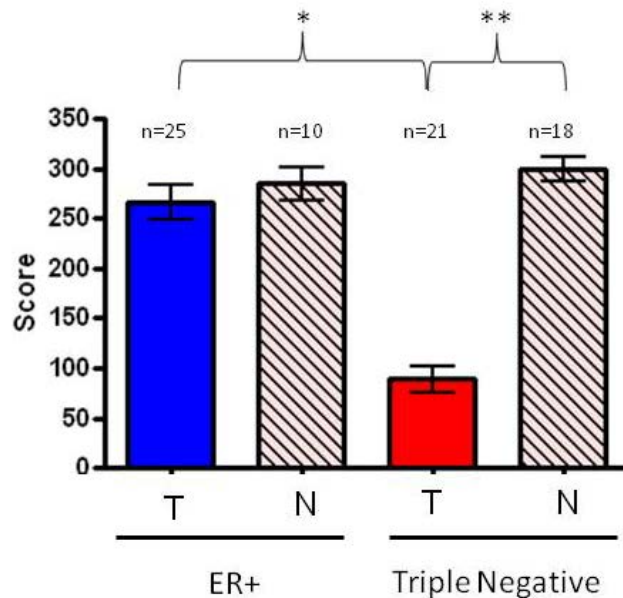
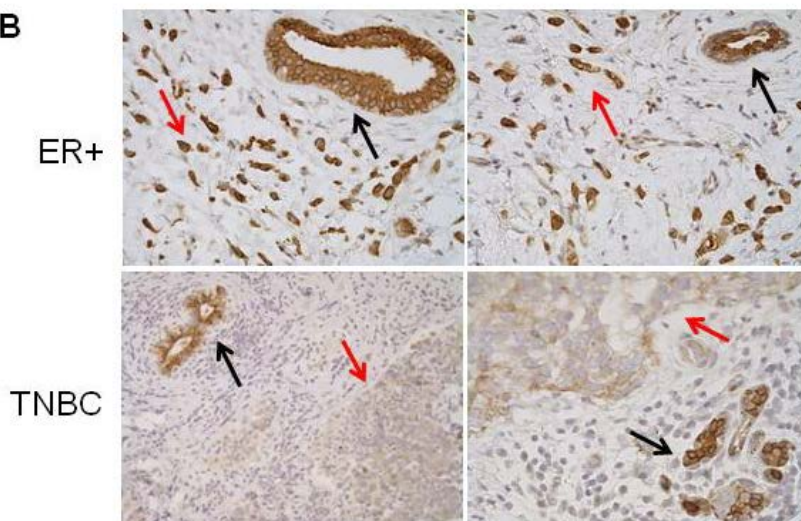


Figure 3. Dicer expression is significantly lower in triple negative breast cancer as compared to adjacent normal breast epithelium, while ER+ tumor levels are not altered. A) ER+ and triple negative breast cancers were stained for Dicer using ab5818, and both tumor and adjacent normal (if present in the tissue section) were scored for Dicer expression by a pathologist. Avg. Dicer score for ER+ and triple negative tumors was 266.2, and 88.9 respectively, and was significantly different between subtypes $*p < 0.0001$, unpaired t test. Avg. Dicer score for adjacent normal in ER+ and triple negative specimens was 285 and 300 respectively. Dicer expression was not significantly different between ER+ tumor and adjacent normal, however, it was much higher in adjacent normal compared to tumor for triple negative cases. $**p < 0.0001$, paired t test, $n = 18$ pairs. B) Representative pictures of tumor (red arrows) and adjacent normal tissue (black arrows) in ER+ and TNBC stained for Dicer using ab5818, 400X.

B

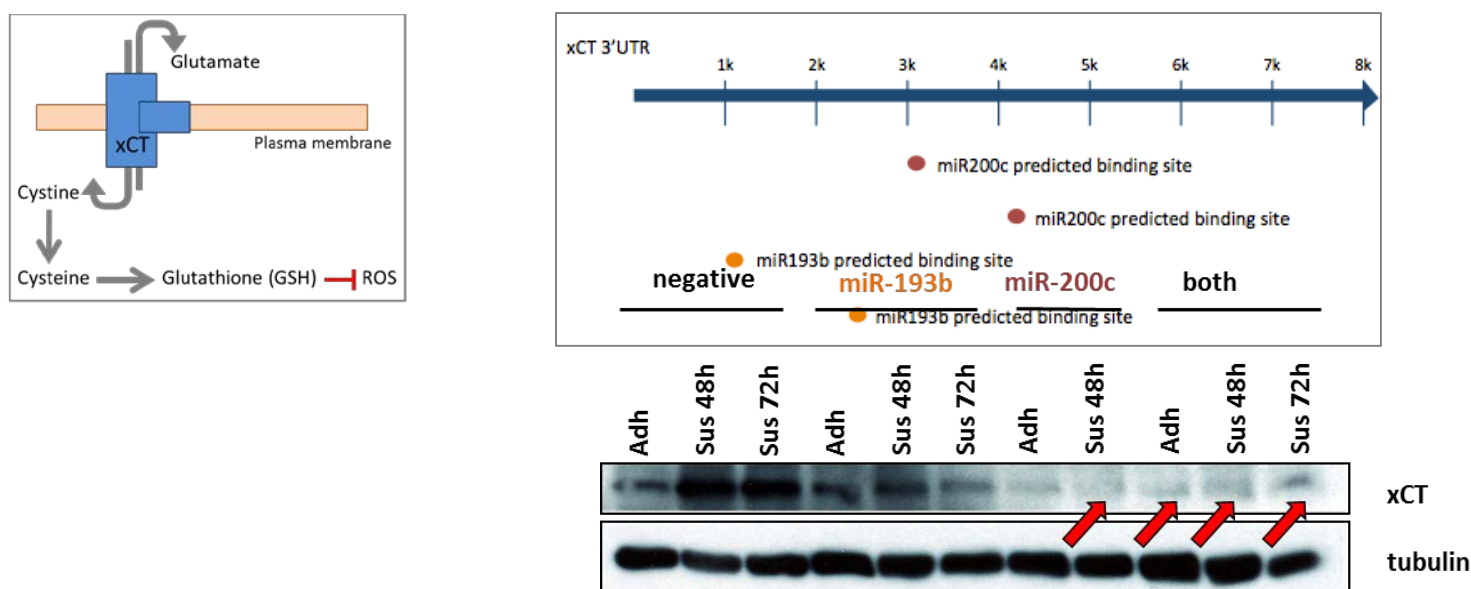


As part of Aim 1 Task 2 we stated that we would determine, at the protein level, genes that increased in suspension and were possible targets of miR-200c. We find that TNBC upregulate regulators of reactive oxygen species such as xCT and stress and inflammatory pathway genes including COX2 and downstream effector levels of prostaglandin E(2) (PGE(2)), which also increased in suspension. Lastly, we have also followed up on

our observation that components of the kynurenine pathway (KP), such as the rate limiting enzyme TDO2 (that leads to production of the essential pyridine nucleotide, nicotinamide adenine dinucleotide (NAD(+))) increase in suspension. A metabolite of this pathway, kynurenine, and the receptor that it binds to, the arylhydrocarbon receptor, also increase in suspension. These factors contribute to the ability of TNBC to resist anoikis and be more metastatic in vivo. We demonstrate that knockdown of COX2 increases death in suspension as does knockdown or inhibition of TDO2 or AhR.

xCT is a glutamine transporter that has recently been found to be very important in TNBC (4) because it regulates intracellular glutathione levels protein. We find that xCT increases in suspension and that restoration of miR-200c decreases the amount of xCT. There are multiple predicted target sites for both miR-200c and miR-193b in the xCT 3'UTR, so we are cloning this region to prove that it is a direct target of these miRNA, since miR-200c is lost in TNBC that are anoikis resistant, and miR-193b decreases in suspension. We find that restoration of miR-200c to TNBC reduces xCT (Figure 4), which regulates intracellular glutathione levels to allow TNBC to cope with reactive oxygen species (ROS). Normally cells die if they can't handle higher levels of ROS, so if we could keep xCT levels reduced, perhaps we would be able to kill TNBC cells. This will be tested next.

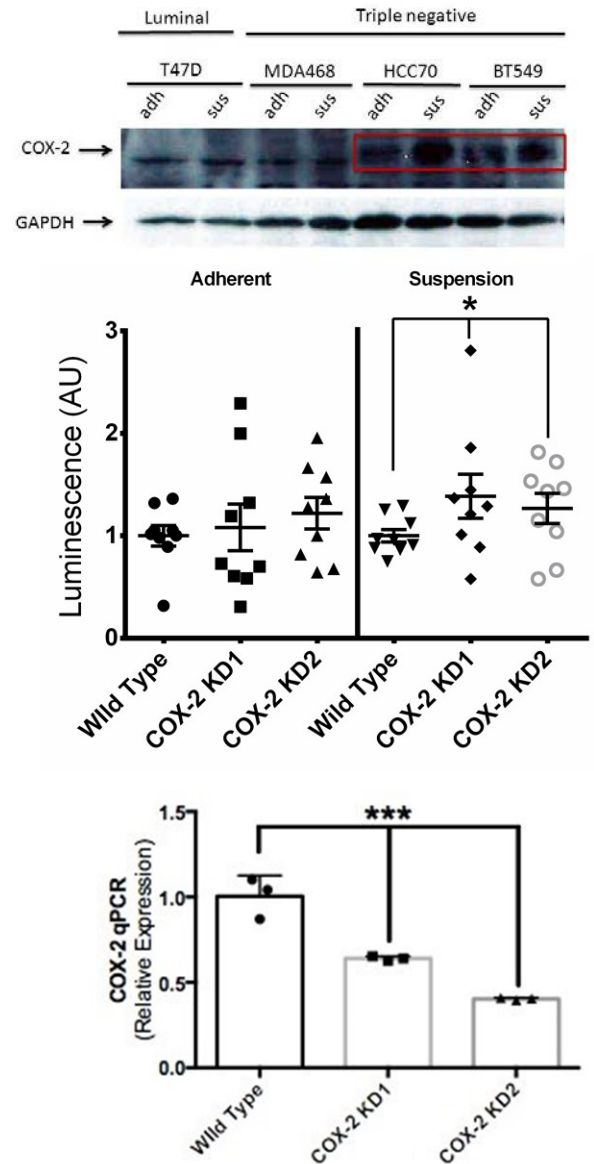
Figure 4. xCT is targeted by miR-200c anoikis resistance. Illustration of xCT's function to reduce ROS. Predicted target sites of miR200c and miR193b on the 3'UTR of xCT (TargetScan). BT549 TNBC cells were transiently transfected with mimics of miR200c, miR193b, or both, then plated in attached or suspended conditions for 48 or 72 hours.



Because Aim 1 Task 2 overlaps with Aim 2 Task 6, I am going out of order a bit in this report, because it was logical for us to do these experiments at the same time and to present them this way. For Aim 2 experiment 1 (Task 6- Months 13-15) We find that stress and inflammatory pathway genes including COX2 and its downstream effector levels of prostaglandin E(2) (PGE(2)) increased in when tumor cells were cultured in forced suspension. Cyclo-oxygenase-2 (COX-2) gene expression increased in our gene expression profiling and the protein increases in suspended conditions in HCC70 and BT549 TNBC cells (Figure 5 top). Prostaglandin E synthase (*PTGES*) also increased in our profiling of BT549 cells attached versus suspended. Interestingly the Weinberg lab published that carcinoma cell-derived interleukin- induce mesenchymal cells of the tumor-associated stroma to induce prostaglandin E(2) (PGE(2)) secretion to induce activation of β -catenin signaling in the cancer cells and formation of cancer stem cells (5). However, we believe that TNBC cells may be able to do this on their own in an autocrine manner when in suspension. In experiment 1 of Aim 2 we said we would determine if inhibition of COX-2 reverses the ability of TNBC cells to resist anoikis. In a collaboration with Dr.

Traci Lyons in the division of oncology at our institution, we tested whether knockdown of COX2 would increase cell death in suspension, and indeed it does (Figure 5 middle and bottom).

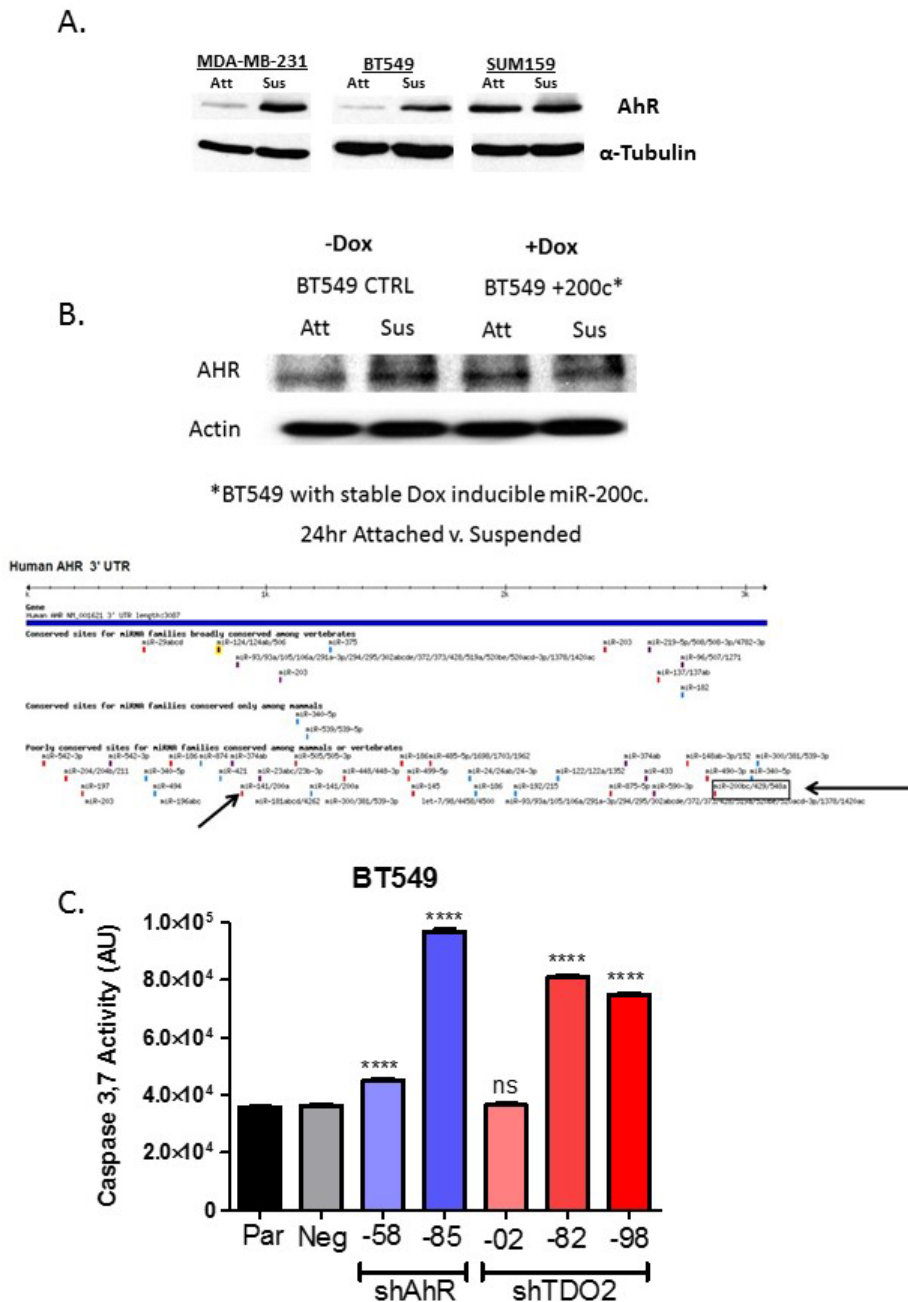
Figure 5. Genes encoding COX-2 and other stress and inflammatory factors increase under conditions of forced suspension and COX-2 protein expression increases in suspended conditions in two of 3 TNBC cells examined. MDA-231, HCC70 and BT549 TNBC lines and the luminal T47D were cultured in attached versus suspended conditions for 48 hrs.. Whole cell extract was harvested in RIPA buffer and probed with antibody recognizing COX-2 (top). In a collaboration with Dr. Traci Lyons in oncology at the Univ. of Colorado, MCF10DCIS human breast cancer cells (Wild type) +/- shRNA for COX-2 (COX-2 KD1 and COX-2 KD2) were grown in adherent or forced suspension conditions (plates were coated with polyHEMA) for 24 hours prior to being incubated with Caspase Glo reagent (Promega) to detect apoptotic cells by luminescence (middle). Data is normalized to wild type and individual data points from 3 independent experiments shown. $P < 0.05$. RT-PCR for COX-2 confirms knockdown with two shRNAs at the transcriptional level.



For Aim 2 experiment 2 (Task 7- Months 15-17) we stated that we would determine if repression of the kynurenine pathway (KP) reverses anoikis resistance in TNBC cells. The KP pathway is the principal route of l-tryptophan catabolism leading to the formation of the essential pyridine nucleotide, nicotinamide adenine dinucleotide (NAD(+)). This pathway has been implicated in immune suppression and tumor progression (6). Interestingly, we observed that 3 genes in this pathway (tryptophan 2,3-dioxygenase (TDO2), kynureninase L-kynurenine hydrolase (KYNU) and the aryl- hydrocarbon receptor (AHR) are among the 10 genes most highly upregulated by TNBC cells under conditions of forced suspension for 24 hrs. Our studies during this reporting period determined that AhR protein in TNBC does increase in suspension culture and restoration of miR-200c reduces this increase in AhR in suspension (Figure 6 top and middle). This likely occurs via 3 binding site,

however, this remains to be tested. Lastly inhibition of AhR via direct knockdown, reduces the ability of TNBC to resist anoikis (Figure 6 bottom).

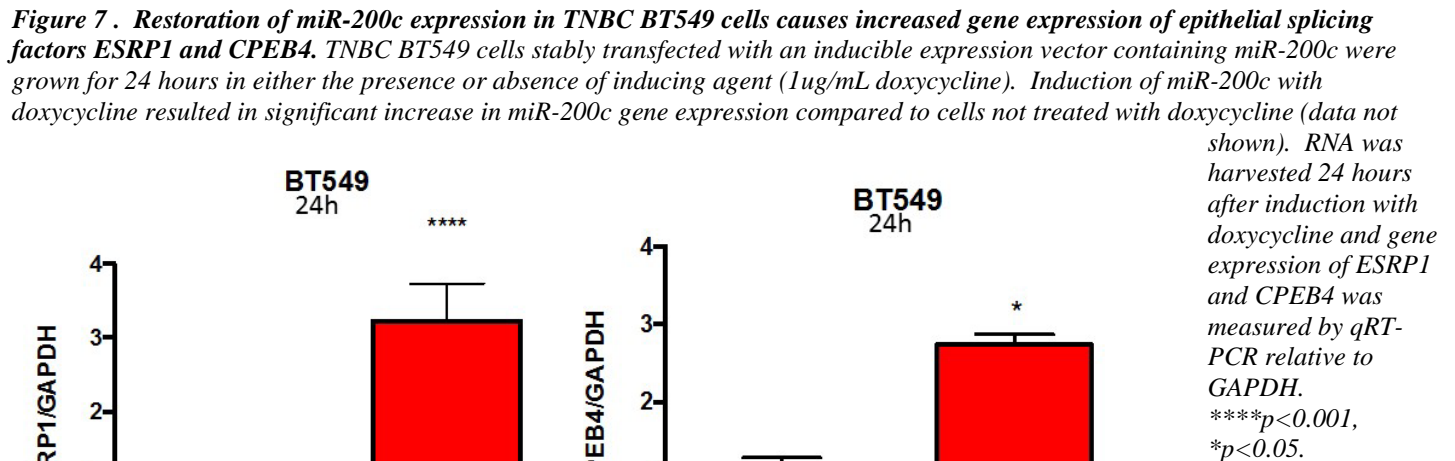
Figure 6. Arylhydrocarbon receptor (AhR) increases in TNBC cell lines in suspension, a process that may be abrogated by restoration of miR-200c and direct knockdown of AhR increases anoikis sensitivity. (A) Colony number of SUM-159 or BT549 cells pre-treated with veh, CH-223191 (10uM), or 680C91 (10uM) for 24 hours, then plated in .25% soft agar in the continued presence of treatment for 10 days. (C) Caspase 3/7 activity in cells pre-treated with veh, CH-223191 (10uM), 680C91 (10uM), or both for 24 hours, then grown in suspension 48h in continued presence of inhibitors. (C) Caspase 3/7 activity in BT549 (left) or MDA-231 (right) cells transduced with non-targeting shRNA (shNEG) or shRNA constructs targeting AhR or TDO2, and plated in poly-HEMA coated plates for 48hrs. * $p < .05$ by t-test; ** $p < .01$, *** $p < .0001$ by ANOVA with Bonferroni's Multiple Comparison Test.



Task 3. Months 5-8. To test whether ER will be restored by antagonizing miR-222 (not yet initiated). We will stain for ER following antagonizing miR-222, since we and others published that ER is directly targeted by miR-222 (1, 7). Determine if tamoxifen or ICI reduces the number and size of colonies in colony formation assays. We have not started this aim yet because we have been following up more on task 2-4), but we will address this next year. We will stain for ER and if ERalpha protein is restored, we will treat cells with or without 10 nM E2 and measure ER regulated proteins progesterone receptors (PR) and PS2.

Task 4. Months 8-10. Identify splicing factors affected by miR-200c. Perform western blots for ESRP1, PTBP1, RBFOX1, RBFOX2. (75% completed). Figure 7 shows results from RT-PCR of two epithelial splicing factors that increase in suspension when miR-200c is induced in BT549 TNBC cells. The mesenchymal splicing

factors RBFOX1, RBFOX2, were not altered (not shown). Next we plan to determine if the cells that have inducible miR-200c and the stable antagomir of miR-222 will do both (increase epithelial splicing factors and decrease the mesenchymal splicing factors that should not be on in epithelial-derived cells (as these carcinoma cells are)). In other words, we can potentially alter the splicing of many genes by altering these miRNAs. We will then perform Next Generation deep sequencing on RNA-seq from these cells or Affymetrix GeneChip Human Exon 1.0 ST arrays to be able to identify specific alternative splicing events.



Task 5. Months 10-12. Perform in vivo experiment to determine if simultaneous restoration of miR-200c and inhibition of miR-222 will render TNBC cell less invasive and more responsive to chemotherapy. (Not yet initiated). We have not yet initiated this experiment as we are still working out the best conditions, but plan to do this in the next year. We state under goals for the next term below, what our plans were and remain as for this experiment. We do have IACUC and ACURO approval for the experiments as well as IRB approval for use of the patient derived tumor xenografts.

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7. Di Leva G, Gasparini P, Piovan C, Nganheu A, Garofalo M, Taccioli C, Iorio MV, Li M, Volinia S, Alder H, Nakamura T, Nuovo G, Liu Y, Nephew KP, Croce CM. MicroRNA cluster 221-222 and estrogen receptor alpha interactions in breast cancer. *J Natl Cancer Inst* 2010; 102: 706-21.

4. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?** This work may have great impact on the field of breast cancer and cancer research and oncology practice eventually, because we have identified genes and their protein products that TNBC make only when they are in suspension. These findings are of interest in cancer biology because we never would have known that the most aggressive TNBC subtype of breast cancer could make these different genes and proteins if we did not do our global screening of what they are making in suspension. The data are different from the data in the TCGA database because those are genes expressed in the primary tumor, not cells that can escape from the primary and survive in transit to a secondary metastatic site. If we identify what tumor cells are using to survive in suspension and if these things are being inappropriately allowed to be expressed because due to microRNA alterations, then can we can potentially use miRs to achieve “differentiation therapy” via altering a whole pattern of gene expression and possibly even alternative splicing events by inducing expression of epithelial splicing factor. Knowledge of these proteins that are facilitating survival in suspension, may provide unique, previously unidentified targets for TNBC therapy.

What was the impact on other disciplines? Our findings and the idea that cancer cells may turn on totally different genes and proteins when they are in suspension is very unique and may be pertinent to other cancers and even in developmental biology.

- **What was the impact on technology transfer?** None to date.

What was the impact on society beyond science and technology? I have given reports of our research to both scientific and lay audiences to improve knowledge regarding how and why triple negative breast cancer is so aggressive and how we are elucidating unique ways to potentially target this subtype of breast cancer that has a high mortality rate, particularly when it becomes resistant to chemotherapy.

- **What do you plan to do during the next reporting period to accomplish the goals?**

In the future we will optimize proper number of cells and other conditions for the 3D matrigel task of staining for polarity proteins. We will also follow up on our finding that miR-200c restoration increases the two epithelial splicing factors by performing RNA-seq in the BT549 cells with and without induction of miR-200c to identify global splicing events. We could alternatively also analyze the RNA hybridized to Affymetrix GeneChip Human Exon 1.0 ST arrays to be able to easily identify specific alternative splicing events. Such events may contribute to why TNBC are so much more clinically aggressive than ER+ disease.

For the in vivo experiments, we are a little behind because we wanted to think about our best strategy. We will do BT549 cell line in 24 mice first, then if we can get tumor from patient derived explants that have been successfully propagated in mice to infect with the miRNAs then we will test their short term survival in the circulation and ability to colonize metastatic sites.

6. CHANGES/PROBLEMS:

- a. **Changes in approach and reasons for change:** We anticipate no changes in our plans. There are no significant problems other than the technicalities that have slowed us down on a couple of the tasks, which we address directly above in the section on “what we will do in the next reporting period.” We are ahead on some tasks that were scheduled for the second year, but behind on Task 5, an in vivo animal experiment that was scheduled to be done in months 10-12 of this year. We decided that some of the mechanistic data is so novel (the fact that TNBC are upregulating these proteins only when in suspension and that they are critical for their ability to survive in suspension) that we should try to get a

manuscript published first on that aspect before we attempt to interfere with the pathway in vivo. In other words, this may be a good publishable unit even before doing in vivo experiments.

- b. **Actual or anticipated problems or delays and actions or plans to resolve them:** none to report
- c. **Changes that had a significant impact on expenditures.** We have not done planned in vivo study yet so we did not spend 100% yet in this first year. We also took student Thomas Rogers with experience in miRNAs instead of a postdoctoral fellow. However, we just hired a fellow with a lot of experience with animal models, so in the next period of the grant we will at her at 50% if possible.
- d. **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.** None to report
- e. **Significant changes in use or care of human subjects.** None
- f. **Significant changes in use or care of vertebrate animals.** None
- g. **Significant changes in use of biohazards and/or select agents.** None

6. REPORTABLE OUTCOMES and PRODUCTS

What opportunities for training and professional development has the project provided?

Cancer Biology Graduate Program doctoral candidate Thomas Rogers presented the following poster presentations at local meetings

- Cancer Biology Training Consortium (Estes Park, CO)
 - 2014- A TDO2-AhR Signaling Axis Is Upregulated in Suspension and Regulates Metastatic Phenotypes in Triple-Negative Breast Cancer
- Student Research Forum (University of Colorado-Anschutz Medical Campus)
 - 2013- Investigating The Role of the Kynurenine Pathway in Anoikis Resistance in Triple Negative Breast Cancer

Lisa Greene, Cancer Biology Graduate Program doctoral candidate also presented posters at these two local meetings:

- **Greene LJ**, Gordon MA, Cochrane D, Richer JK. Mechanisms of anoikis resistance in ovarian and breast cancer. 2014 Cancer Biology Annual Retreat and Cancer Biology Training Consortium Annual (CABTRAC) Meeting. Estes Park, CO. September 2014.
- **Greene LJ**, Gordon MA, Cochrane D, Richer JK. Mechanisms of anoikis resistance in breast and ovarian cancer. 2014 Cancer Biology Program Retreat, Winter Park, CO. September 2014.

a. How were the results disseminated to communities of interest?

Graduate student Thomas Rogers will be presenting the following abstract as a poster at the SABC: This abstract is provided in the appendix.

- San Antonio Breast Cancer Symposium (San Antonio, TX) Abstract #: 850223
Defining the Role of the Kynurenine Pathway in Mediating Anoikis Resistance in Triple Negative Breast Cancer

Dr. Richer was invited to speak at an international meeting and laboratory in Florence, Italy March 26-28 2014. For the meeting I talked about what we have learned regarding anoikis resistance in both breast and ovarian cancers since TNBC and serous ovarian cancers have many similarities including some of the things we have found to be important for survival in suspension, which is likely why these two diseases metastasize so readily.

Richer, JK. Invited Symposia Lecture “Functional Significance of miRNAs Contributing to Reproductive Cancers” Society for Gynecologic Investigation. Florence Italy, March 2014.

Richer, JK visiting lecturer “Anoikis Resistance in Breast and Ovarian Cancer Progression” University of Florence Dept. Experimental and Clinical Biomedical Sciences, Biochemistry, Human Health Medical School Guest of Professor Paola Chiarugi March 2014.

Dr. Richer also gave a talk for the University of Colorado Cancer Center on this research:

2014 March 21 Cancer Center Hormone Related Malignancies Retreat –“Mechanisms of Anoikis Resistance During Breast and Ovarian Cancer Progression”

Funding applied for based on work supported by this award:

I applied for and was awarded a Breakthrough Award Level 2 grant “Targeting tryptophan catabolism: a novel method to block triple negative breast cancer metastasis.” I had noted that some of the genes mentioned in this grant as potential targets of miR-200c were all in the tryptophan catabolism pathway and a metabolite of that pathway, kynurenine, was recently found to be a ligand for the arylhydrocarbon receptor (AhR), which we also find to be likely targeted by miR-200c (see this report) and is increased in suspension. Interestingly, AhR is not only found in the tumor cells themselves, but is also found in immune cells. Kynurenine (KYN) can act as an immunosuppressive agent by binding to AhR in various immune cells. Thus the **Breakthrough Award goal** is to test the new hypothesis that TNBC can make this ligand (KYN) that can act in a paracrine fashion to effectively shut down the immune system to allow the TNBC to evade immune attack. With a team of collaborators, including a tumor immunologist and an oncologist that specializes in young women’s breast cancer, we will test our theory. The initial confirmation that the components of the tryptophan catabolism pathway, such as the rate limiting enzyme TDO2, were up not only at the gene level, but also the protein level when TNBC are cultured in forced suspension and that it and AhR had miR-200c predicted binding sites came from this Idea Expansion grant. However, at the time that we wrote the idea expansion grant we were only thinking of autocrine effects of KYN, not its effects on the immune system that could potentially explain why TNBC is such an aggressive subtype that often recurs and is lethal within 5 years of diagnosis. The fact that this pathway is targetable makes this particularly exciting and if inhibiting this pathway can render the immune system more able to recognize and effectively attack TNBC tumor cells, then it is a good target for therapy on more than one front.

PRODUCTS:

Journal publications. We have 3 publications **in preparation** that will all be submitted soon.

Nicole S. Spoelstra, Diana M. Cittelly, Anthony Elias, Paul Jedlicka, Jennifer K. Richer. Dicer Expression in Estrogen Receptor Positive vs. Triple Negative Breast Cancer: An Antibody Comparison. In preparation.

Rogers, T., D’Amato, N., Gordon, M., Greene, L., Cochrane, D., Nemkov, T., Spoelstra, N., Hansen, JK. Richer JK. A TDO2-AhR Signaling Axis Is Upregulated in Suspension and Regulates Metastatic Phenotypes in Triple-Negative Breast Cancer. In preparation, 2014

Greene LI, Gordon MA, Cochrane D, Richer JK. Mechanisms of anoikis resistance in ovarian and breast cancer. In preparation 2014

Inventions, patent applications, and/or licenses. None

▪ **Other Products**

We have the inducible miR-200 BT549 cells now (TripZ lentiviral vector) with stably introduced miR-222 miR-ZIP.

▪ **biospecimen collections;**

We have formalin fixed paraffin embedded TNBC lines in culture in attached versus suspension culture.

We have two TNBC patient derived xenograft tumors.

- research material: we have many primers and antibodies now to the genes/proteins of interest.

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7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on the project?

- i. Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Personnel	Role	Percent Effort	Nearest Person Month Worked	Contribution to Project	Funding Support: DOD Contract W81XWH-13-0222
Jennifer Richer, PhD	Partner Principal Investigator	25%	3	Oversees all experiments –, writes reports and edits manuscripts	X
Ann Jeans	Technician	40%	1.2	Performed western blots and tissue culture until she left the department in December, 2013	X
Kiel Butterfield	Technician	100%	2	Since her hire in the lab in June 2014, performs tissue culture, support for attached versus suspended experiments,	X
Thomas Rogers	Graduate Student	100%	2	Began work on this grant in August, 2014, has performed experiments to test for alterations in TNBC cells following miRNA manipulations	X

				and suspen- sion culture	
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- ii. **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?** No changes in active support for the PD/PI(s) or senior/key personnel.
- b. **What other organizations were involved as partners?** None

8. SPECIAL REPORTING REQUIREMENTS - none

9. APPENDICES:

The attached appendix contains Abstract for a 13poster that will be presented by graduate student Thomas Rogers at the San Antonio Breast Cancer Meeting.

Title: **Defining the Role of the Kynurenine Pathway in Mediating Anoikis Resistance in Triple-Negative Breast Cancer**

Authors: Thomas Rogers¹, Nicholas D'Amato¹, Travis Nemkov², Lisa Greene¹, Michael Gordon¹, Kirk Hansen², and Jennifer Richer¹

¹Department of Pathology, University of Colorado-Anschutz Medical Campus, ²Department of Biochemistry and Molecular Genetics, University of Colorado-Anschutz Medical Campus

Background: Anoikis resistance is thought to be a critical trait of metastatic cancer cells, enabling them to leave the primary tumor and travel through extracellular matrix, intravasate, and survive in the vasculature or lymphatics in transit to a metastatic site. This is particularly important for the triple-negative breast cancer (TNBC) subtype, which has a peak risk of recurrence within the first three years post-diagnosis and an increased mortality rate in the first five years as compared to other subtypes. We performed global profiling of TNBC cells in attached versus forced suspension culture conditions (using poly-HEMA coated plates) for 24 hours. These data revealed that TNBC cells surviving in suspension upregulate multiple genes involved in tryptophan catabolism, also known as the kynurenine pathway (KP), including the rate limiting enzyme tryptophan 2,3,-dioxygenase (TDO) and kynureninase (KYNU). A key metabolite of this pathway has been found to activate the aryl hydrocarbon receptor (AhR), which was also up-regulated in suspended cells.

Hypothesis: We hypothesize that the ability to upregulate kynurenine pathway (KP) facilitates TNBC cell survival in suspension and mediates the migratory/invasive potential of TNBC.

Methods: We assessed mRNA and protein levels of TDO2, KYNU and AhR by RT-qPCR and western blot. AhR luciferase reporter activity, as well as known AhR regulated genes, were measured in suspension compared to adherent conditions. TNBC cells were treated with small molecule inhibitors of AhR and TDO2. Additionally, secretion of endogenous kynurenine was measured by high performance liquid chromatography (HPLC). Purified kynurenine was added to rescue AhR activity following TDO2 inhibition. Finally, anoikis sensitivity and migratory potential were measured following pharmacological inhibition of TDO2 and AhR.

Results: Relative mRNA levels of TDO2, KYNU, and AhR increase 9, 7, and 2 fold respectively in suspended TNBC cells compared to adherent conditions ($P < 0.0001$). Estrogen receptor positive breast cancer cells lines do not significantly upregulate these genes. AhR reporter activity ($P < 0.0001$) and nuclear localization increase in suspended TNBC cells. Additionally, AhR reporter activity ($P < 0.0001$) and AhR target gene expression (CYP1A1, CYP1B1: $P < 0.0001$) decreased in TNBC cells treated either AhR antagonist (CH223191) or selective TDO2 inhibitor (680C91). Conversely, when purified kynurenine was added to TNBC cells in vitro, AhR activity increased ($P < 0.05$). Furthermore, kynurenine secretion, as measured by HPLC, increased 2.5 fold in suspended TNBC cells and this increase in kynurenine was reduced by addition of 680C91 ($P < 0.0001$). Finally, targeting TDO2 or AhR increased anoikis sensitivity and decreased migration of attached and suspended TNBC cells.

Conclusions: Collectively, these results suggest that the kynurenine pathway may play a critical role in metastatic TNBC. Further mechanistic studies will focus on how the kynurenine pathway is mediating these tumorigenic properties either through the de novo synthesis of NAD⁺ and/or activation of AhR by kynurenine. Targeting the kynurenine pathway in the clinic may provide therapeutic strategy that could dramatically reduce TNBC mortality rates.

Funding: We wish to acknowledge Department of Defense Breast Cancer Research Program DOD BC122994 W81XWH-12-BCRP-IDEX to JKR